Attorney Docket No. 550-128
PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re Application of:
LEWIS et al.

Serial No.: 09/284,009 Group Art Unit: 1633

Filing Date: April 5, 1999 Examiner: Sorbello, E.

Title: MONONUCLEAR PHAGOCYTES IN THERAPEUTIC DRUG DELIVERY

DECLARATION OF STUART NAYLOR, PH.D.
SUBMITTED PURSUANT TO 37 C.F.R. § 1.132

Commissioner for Patents
Washington, D.C. 20231

Sir:

1. I am Vice President: Biological Systems at Oxford BioMedica, the assignee of the present application.

2. Reference may be had to my *curriculum vitae*, attached hereto as Appendix A, for detailed information concerning my education and work experience. Briefly, I received my Bachelor of Science degree in Microbiology and Virology in 1984 from University of Warwick and Ph.D. in Tumour Biology in 1992 following completion of studies at the Imperial Cancer Research Fund. After I obtained my doctorate, I spent a further 5 years in postgraduate study at two of the top Cancer Institutions in Europe: the Imperial Cancer Research Fund and the Institute of Cancer Research, specializing in the role of cytokines [illegible]

[illegible] Page 1 of [illegible]

numerous publications in peer reviewed journals in this field. More specifically at ICRF I spent 4 years focused on the role of macrophages in the tumour microenvironment with reference to migration, tumouricidal activity and matrix remodeling (see refs attached). I am currently Vice President: Biological Systems at Oxford BioMedica with primary responsibility for the company's macrophage-based gene delivery programme (MacroGen).

3. The patent application is premised on the important and unexpected finding that mononuclear phagocytes modified to comprise at least one hypoxia and/or ischemic and/or stress regulatable element operably linked to at least one nucleotide sequence of interest (NOI) may be used in methods of targeting a mononuclear phagocyte to hypoxic and/or ischemic and/or stress sites, for example for the treatment of cancers.

4. The following data is provided demonstrating intra- and peri-tumoural administration of adenovirally transduced macrophages into established MDA 231 tumour xenografts.

Example I

Macrophage preparation

Macrophage culture and transduction was carried out as described in the instant specification, Example 2 (page 23 line 23 to page 25 line 17).

Adenoviral Vector Preparation

The adenoviral vector preparations used were an adenoviral vector comprising a CMV promoter and a green fluorescent protein reporter gene (Ad CMV GFP) and an adenoviral vector comprising a hypoxic response element (HRE) and a LacZ reporter gene (Ad HRE Lac Z). Ad HRE Lac Z is the same adenoviral vector as referred to in the instant specification as QB1-HRE-LacZ and as shown in Figure 4 of the specification. These vectors displayed [illegible]

Adenoviral transduction of macrophages

The adenoviral vector transduction efficiency was routinely >80% as monitored by UV microscopy in Ad CMV GFP transduced cells.

MDA 231 tumour xenograft preparation in mice

Two 0.5cm diameter MDA 231 sub-cutaneous tumours were established on nu/nu mice.

Transfer of adenoviral transduced macrophages into mice with MDA 231 tumour xenografts

Approx $2x10^6$ macrophages ($1x10^6$ per tumour) were administered subcutaneously around the periphery of the tumour or intratumourally in a volume of 100µl per tumour. After three days the tumours were removed and immersed in formalin. The samples were paraffin wax embedded, sectioned and immunostained for GFP protein. Samples injected with untransduced macrophages were used as a negative control.

Subcutaneous Administration

Subcutaneous injections were given around the periphery of the tumour (i.e. peritumourally) and great care was taken to avoid the needle penetrating the tumour mass.

Figure 1 shows strong positive staining for GFP in cells with macrophage morphology in the tumour xenograft injected with macrophages transduced with Ad. CMV GFP (red indicates positive staining).

Figure 2A shows in the left hand column, sections of tumours injected with untransduced macrophages and immunostained for Lac Z (control).

Figure 2B shows in the right hand column, sections of tumours injected with Ad.HRE. LacZ macrophages and immunostained for LacZ.

It is clear from a comparison of Figure 2A (x60)(control) with Figure 2B (x60)(test) that there is evidence for switch on of the HRE promoter in macrophages transduced with Ad. HRE LacZ (i.e. QB1-HRE-LacZ) in the tumour xenograft as positive staining for the LacZ reporter gene is observed. It is also clear from Fig 2B (x60) that the macrophages are located in a necrotic region where an hypoxic environment is highly likely (brown indicates positive staining for macrophages).

In summary, Figure 2(B) (x 60) of the data provided clearly illustrates that the present claimed invention provides a method for selectively localising a mononuclear phagocyte at a target hypoxic tumour site. Accordingly, it would have been routine for the ordinarily skilled person to replace the reporter gene in the Ad. HRE.LacZ construct used in Figure 2(B) and in the specification at Example 2 with for example, a gene which is cytotoxic for macrophages such as a suicide gene (see page 12, lines 19-23) (e.g. to selectively destroy the mononuclear phagocyte according to the method of claim 69). In particular, see WO95/21927 (referenced on page 9 line 2 and page 42 line 15 of the specification), now issued in the US as US Patent 6,265,390, which taught the construction of HRE-marker gene constructs and HRE-prodrug constructs, and the use thereof for hypoxically-regulated expression in tumour cells. Furthermore, the instant specification exemplifies the construction of adenoviral vectors comprising HRE-lacZ and adenoviral vectors comprising HRE-IL2 in Example 2, for instance.

Example 2

Intraperitoneal Studies

As indicated above, macrophage culture and transduction was carried out as described in the instant specification, Example 2 (page 23 line 23 to page 25 line 17).

Human intra-peritoneal ovarian cancer models

Three human intra-peritoneal ovarian cancer models were used. These were entitled IGROV, Ali and HUA.

Ali and HUA models were maintained by *in vivo* passage of human tumour cells in nude mice. IGROV tumours were generated from cell implants. Two weeks after passage the animals had established intraperitoneal tumour masses.

Transfer of adenoviral transduced macrophages into mice with ovarian cancer

Macrophages transduced with Adenoviral CMV LacZ CMV GFP vector were injected in a volume of 200µl intraperitoneally. Three days later the tumours, hearts livers, spleen and lungs were removed, snap frozen, cut and mounted using a cryostat and stained for LacZ expression by X-Gal histochemistry.

Results 2

Figure 3 illustrates the staining results obtained for IGROV, HUA and Ali tumour xenografts after intra-peritoneal administration of macrophages. The blue precipitate indicates positive staining for Lac Z. Co-localisation with both GFP and CD68 (which is a macrophage marker) immunohistochemistry was evident.

Summary

The above described results clearly support that:

(i) an adenoviral transduced macrophage can deliver a nucleotide sequence of interest (NOI) to a target site; and

(ii) the NOI delivered by adenoviral transduced macrophage can be selectively expressed at a target site such as a hypoxic site when the expression of the NOI is regulated by a hypoxic response element (HRE).

The data described here in Examples 1 and 2 clearly support that the methods and results described in the present claimed invention are enabling in an in vivo situation without undue experimentation and that one of ordinary skill in the art would have been able to make and use the claimed invention at the time the claimed invention was made. As mentioned above, the replacement of a reporter gene with a therapeutic gene would have been a matter of routine experimentation for one of ordinary skill in the art. In particular, see WO95/21927 (referenced on page 9 line 2 of the specification), now issued in the US as US Patent 6,265,390, which taught the construction of HRE-marker gene constructs and HRE-prodrug constructs, and the subsequent use thereof for hypoxically-regulated expression. Furthermore, the instant specification exemplifies the construction of adenoviral vectors comprising HRE-lacZ and adenoviral vectors comprising HRE-IL2 in Example 2, for instance.

Although the vectors chosen for the present exemplification are adenoviral vectors, the above data may be extended to any viral vector capable of transducing a monocyte/macrophage. These vectors include an adeno-associated viral (AAV) vector, a herpes-virus vector or a retroviral vector such as a lentiviral vector (see page 14, lines 5-22 of the application as filed).

As further evidence in support of the presently claimed invention, I submit the following:

It is well known in the art that hypoxia is a powerful regulator of gene expression in a wide range of different cell types (Wang and Semenza 1993 Proc Natl Acad Sci USA 90:4304-

hypoxia inducible factor-1 (HIF-1), which bind to cognate DNA recognition sites, the hypoxia-response elements (HREs) on various gene promoters such as GAPDH and VEGF thus upregulating the expression of that gene.

The present application teaches on page 15, lines 3-6 that:

"The enhancer may contain elements for regulated expression such as a hypoxia regulated enhancer (for example a binding element for the transcription factor HIF1) or elements which respond to stress or low glucose"

and it teaches on page 25, lines 22-24 that:

"Sequences from a region approx, 300-375bp upstream of the transcription start of the human Enolase A gene were chosen containing three HIF-1 consensus binding sites (Semenza *et al* 1996 J. Biol. Chem. 271: 32529-32537".

In the attached Figures 4, 5 and 6, we demonstrate that macrophages express HIF-1α when exposed to hypoxia *in vitro* or in avascular areas of human tumours (Figure 4), human wounds (Figure 5) and human arthritic joints (Figure 6).

In this respect, Figure 4 shows that Macrophages express HIF-1α when exposed to hypoxia *in vitro* or in avascular areas of human tumours. The figure shows immunolocalization of HIF-1α in human monocyte-derived macrophages (A,B) following exposure to either normoxia (20.9% O_2; A) or hypoxia (0.5% O_2; B) for 16 hours *in vitro*. Immunoreactive HIF-1α is seen in both the cytoplasm and nuclei (arrows) following hypoxic induction. Also shown is expression of immunoreactive HIF-1α by CD68-positive macrophages in a human ovarian (C,D) and breast (E,F) carcinoma. Sequential 3μm sections of wax-embedded tumours were immunostained for the pan-macrophage marker CD68 (C,E) and for HIF-1α (D,F). HIF-1α is present in both the cytoplasms and nuclei of

Figure 5 shows that macrophages express HIF-1α in human wounds. The figure shows immunolocalization of HIF-1α by macrophages in a human dermal wound, two weeks post-injury (A,B) and an ovarian carcinoma (C,D). Macrophages (A,C) are highlighted in red using a monoclonal anti-human CD68 coupled to an alkaline phosphatase/Fast Red staining method, and HIF-1 (B,D) in brown using a monoclonal anti-human HIF-1α coupled to a peroxidase/DAB staining method. Scale bars = 50μm.

Figure 6 shows that macrophages express HIF-1α in human arthritic joints. The figure shows immunohistochemical localization of CD68-positive macrophages (brown colour reaction using a peroxidase substrate, top left), HIF-1α (red colour reaction using an alkaline phosphatase substrate, top right) and CD31-positive microvessels (brown, bottom left) in the hypoxic, outer intimal layers of human synovial tissue from a joint with rheuamtoid arthritis.

Summary

Figures 4-6 therefore provide evidence that hypoxic conditions can induce the activity of hypoxia inducible transcription factors such as hypoxia inducible factor-1 (HIF-1) (Wang and Semenza 1993 *ibid*), which is capable of binding to cognate DNA recognition sites, the hypoxia-response elements (HREs) and upregulating the expression of a gene associated with the HRE.

The data provided herein clearly supports that a mononuclear phagocyte that has coupled thereto, or internalised therein, a hypoxia and/or ischemic and/or stress regulatable agent can localise and express a gene of interest at a target site.

Accordingly, all claims are enabled by the present application as filed.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 24th April 2002

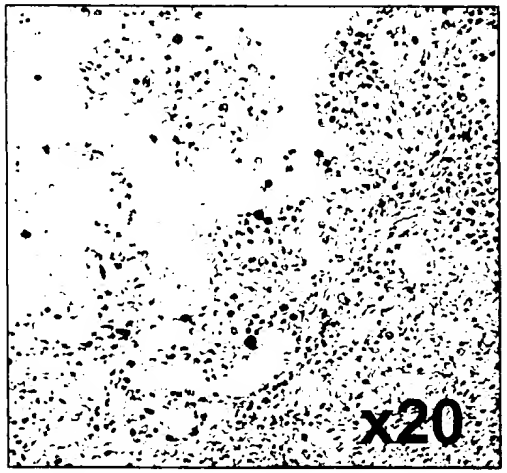
Stuart Naylor, Ph.D.

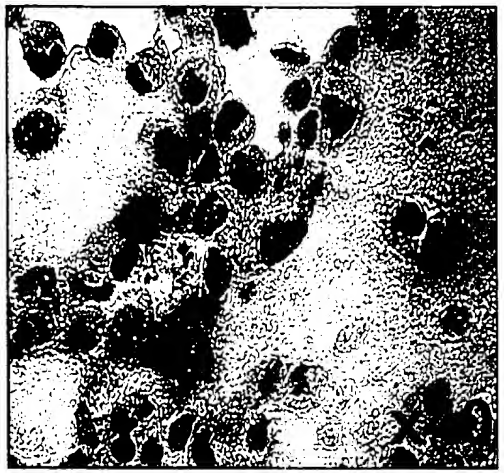
H:\Mem\obm\obm 15\stu OBM15US declaration (final version 22 apr 02).doc

Peri-tumoural injection of Adenovirally transduced macrophages into MDA 231 xenografted tumours

Fig 1

Ad. CMV GFP macrophage injected tumour sections immunostained for GFP protein- red is positive



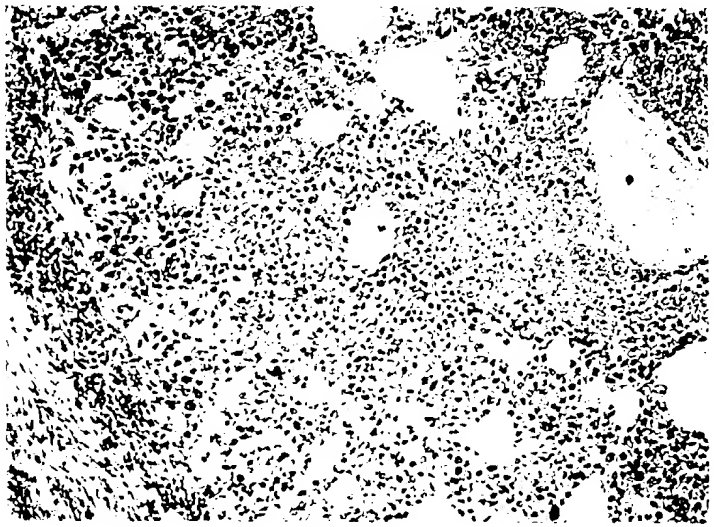


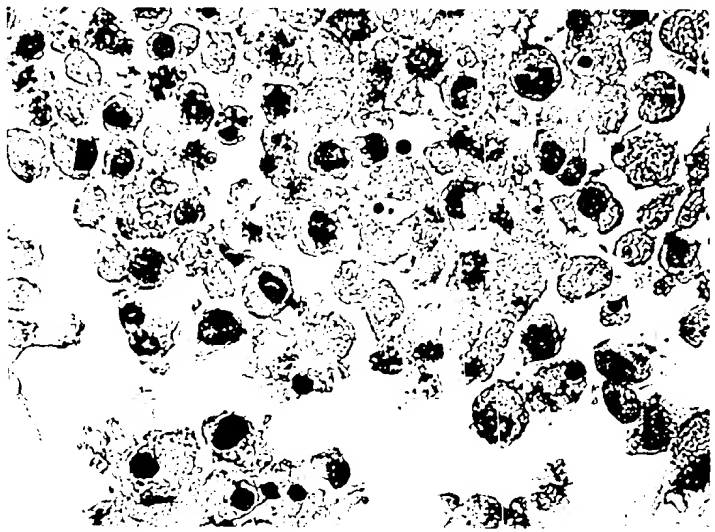
Sections of tumours injected with **untransduced macrophages** and immunostained for LacZ **Fig 2A**

Sections of tumours injected with **Ad. HRE Lac Z macrophages** and immunostained for LacZ **Fig 2B**

x10

x60





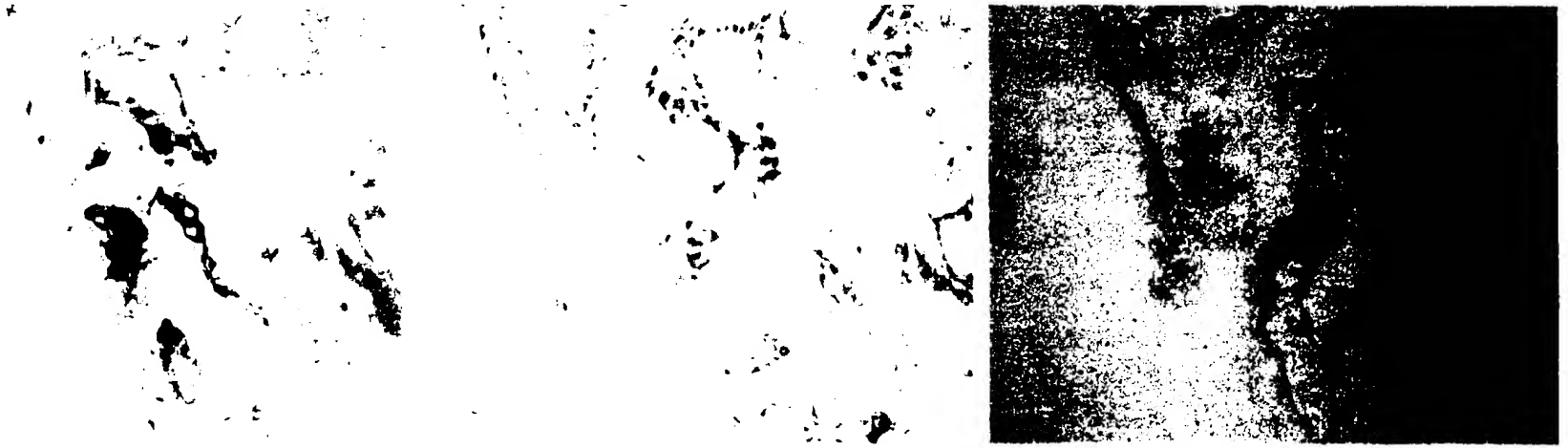
+ve

Intraperitoneal Injection of Ad.CMV GFP CMV LacZ Positive Macrophages

Fig 3

IGROV Tumours - Foci of macrophages located at tumour periphery

HUA Tumours - Macrophages clustering around the periphery of tumour nests

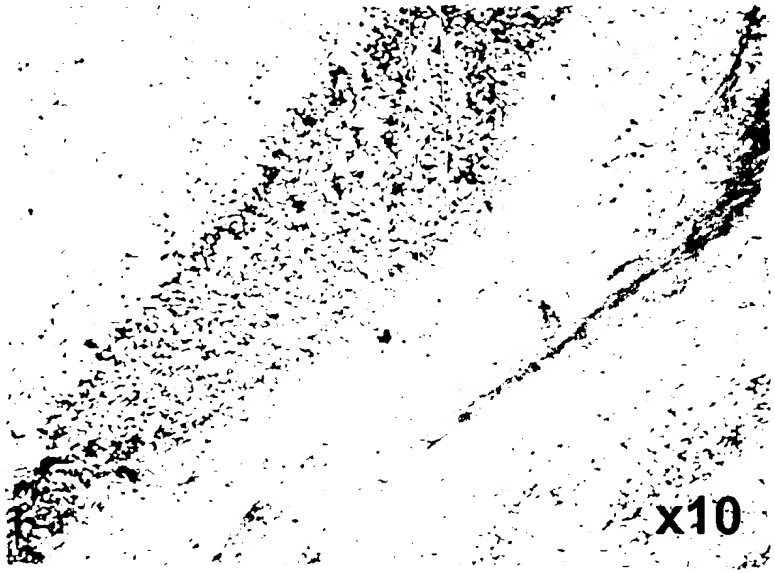


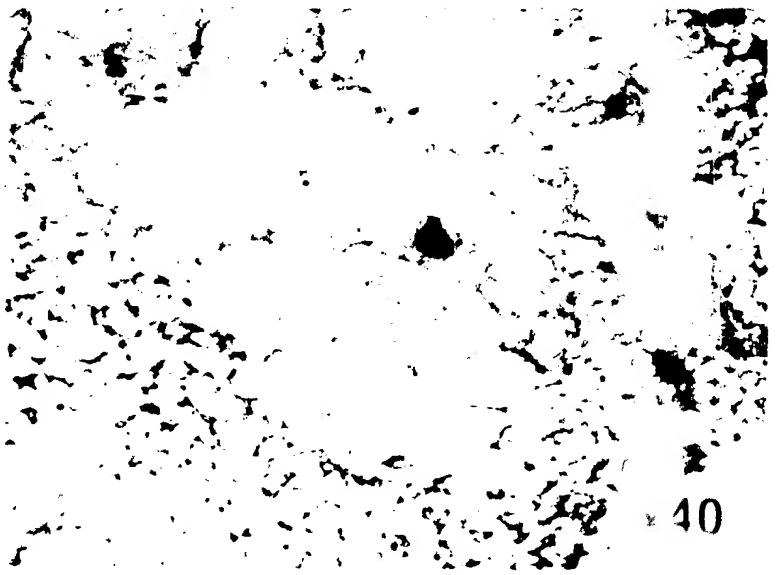
X-Gal (histochemical stain for LacZ - positive is blue)

CD68 (Macrophage marker) positive is brown

GFP-positive is br

Ali Tumours - Macrophages locating to intratumoural necrotic sites Fig 3





X-Gal (histochemical stain for LacZ - positive is blue)

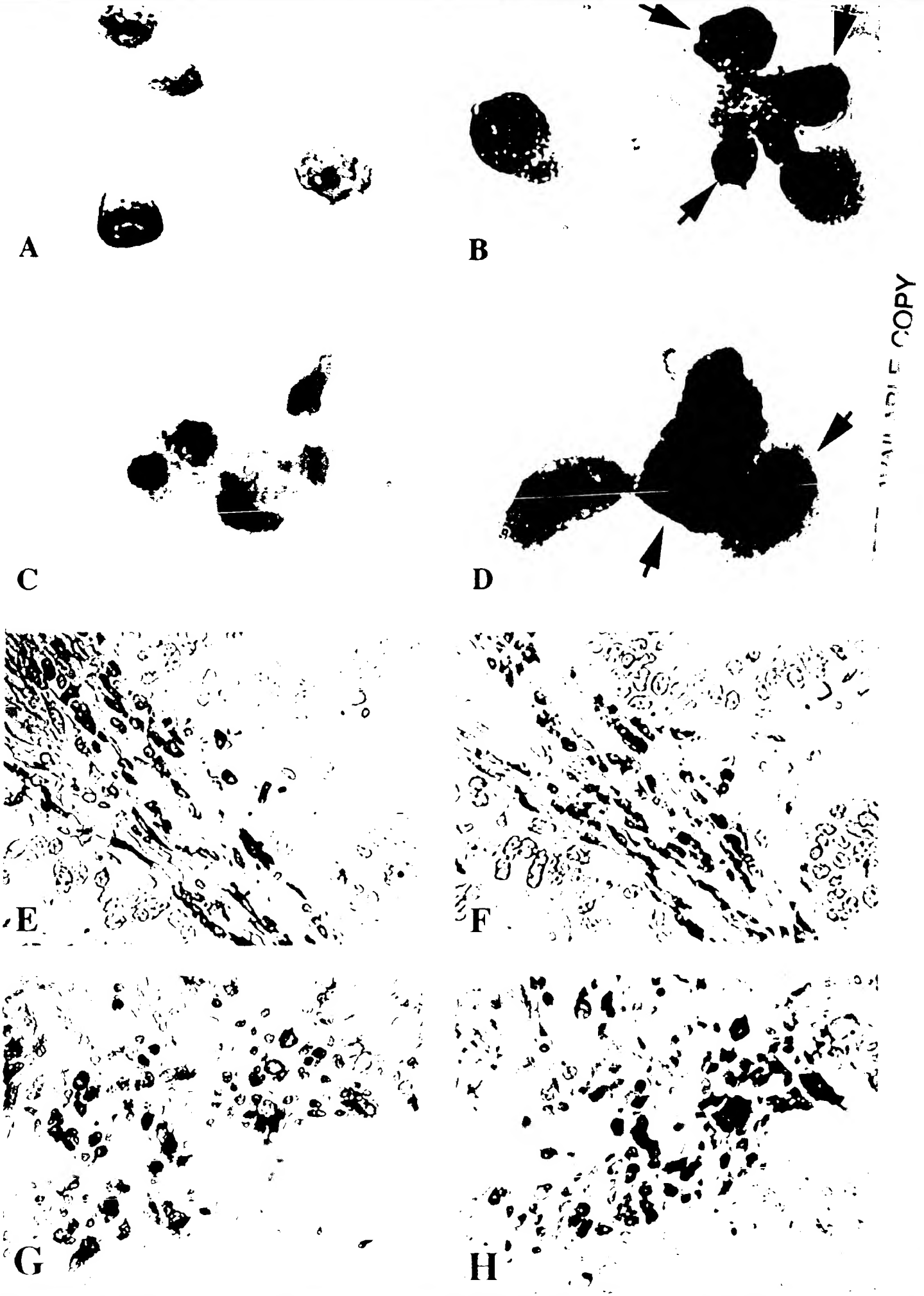


FIGURE 4

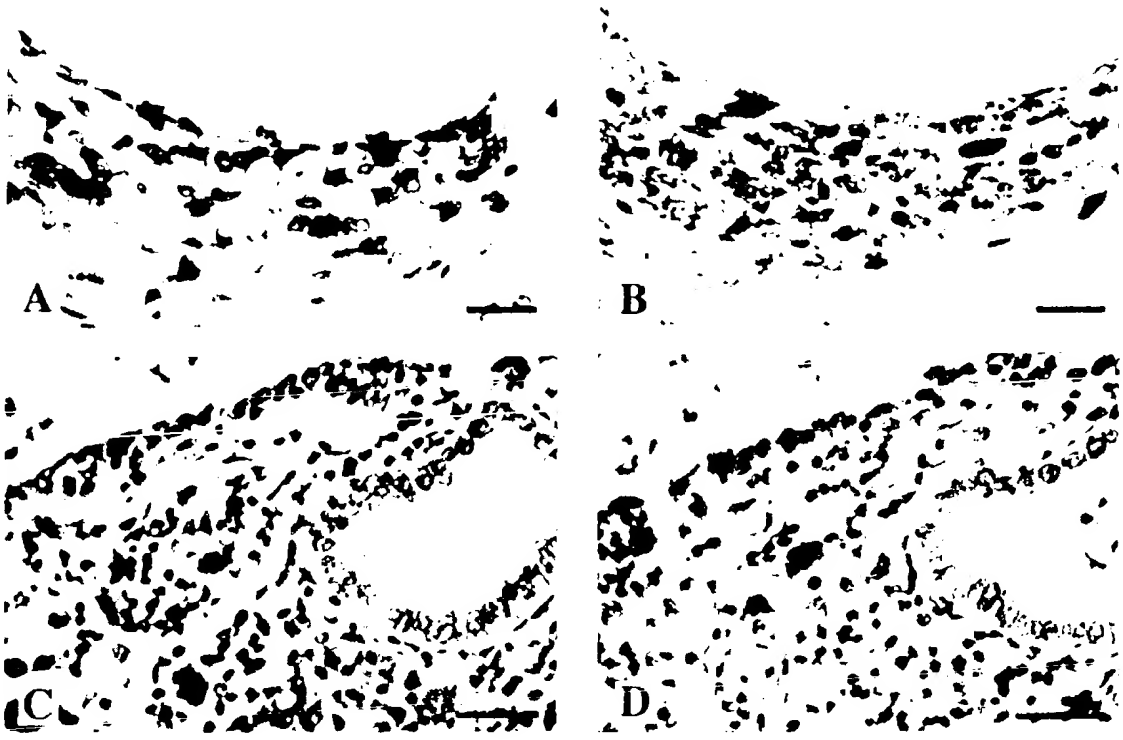


FIGURE 5

Appendix A
(Michael) Stuart Naylor PhD

64. Woodside Road
Amersham
Bucks.
HP6 6AN

Tel (home) 01494 723898
Tel (work): 01865 783000
Email s.naylor@oxfordbiomedica.co.uk

Date of Birth 9th April, 1963
Married (two children)

Profile

Highly motivated, energetic and enthusiastic person with excellent 'people' skills whose key goal is to inspire colleagues and develop a framework for success

---

Career History

Oxford BioMedica **April 1997-present**
Oxford Science Park
Oxford BioMedica
OX4 4GA

April 1997-Jan 1998 Senior Scientist

Jan 1998-present:

Current Position

Director: Biological Systems Group (formerly known as Tumour Biology)

- Reporting to the Board via the Senior Vice President of Research

Key responsibilities

- Head of Biological Systems Group comprising of 10 research scientists covering projects in gene therapy (cancer /cardiovascular) and gene discovery (functional biology/validation). In addition shared supervision of a BBSRC student at the Royal Holloway and a Post-Doctoral Scientist at ICRF

- Member of Executive Research Group the Company body overseeing all research activities and strategy

- Programme leader for two key commercial collaborations: Aventis and IDM (in expansion)

- Primary responsibility for the clinical development of three novel gene therapy product families:

  MetXia, a GDEPT strategy for the treatment of cancer (currently in Phase I/II for breast and ovarian)
  ProCaStat, a GDEPT strategy for the treatment of prostate cancer (in clinical development, Phase I/II 2002)
  MacroGen, a cell based gene delivery strategy for the treatment of intraperitoneal cancer (Phase I/II 2002)

- Extending clinical network
  Setting up and maintaining close relations with clinicians to extend our clinical network and generate a group of advisors to assist in shaping clinical development

- Extending academic network
  Identify collaborative opportunities for translational research

- Extending Intellectual Property portfolio

- Extending research profile through presentations and publications

- Commercialisation of OBM technologies and products in collaboration with SVP Business Development through preparation of marketing documents and commercially biased presentations of OBM technologies and clinical products

Key achievements:

- Generation and compilation of data for key commercial presentation leading to secure the biggest commercial collaboration to date (Aventis-RPR). Underpinned the second round of fund raising to ensure the continued growth of the company

- Establishment of core research projects/IP in gene discovery leading to a targeted fund raising for the creation of a new Division within the company. Division of Gene Discovery

- Driving preclinical development of MetXia to enter the clinic in an accelerated time frame

- Taking cell-mediated gene delivery from conception to the clinic

- Developing *de novo* the infrastructure to perform cardiovascular disease research

- Highest scientific publication rate of any group in the company

- Presentation leading to a collaboration with Astra Zeneca on target validation

- Extension of the Company

Institute of Cancer Research Oct 1993-April 1997
Haddow Laboratories. Royal Marsden Hospital
Sutton. SURREY SM2 5NG

Senior Postdoctoral Scientist:
Developmental Biology Team. Section of Breast Cancer Biology (Head: Barry Gusterson)

Development of the transgenic fat pad model for assessing the role of the Wnt gene family in the development of the mammary gland.
Developed new retroviral vectors for *ex vivo* transduction of primary mammary epithelial cells. these were then transplanted back into surgically cleared mammary fat pads of mice and their impact on development analysed. Novel retroviral vector genomes were constructed, primary cell culture was optimised and techniques to analyse gene expression in reconstituted glands were established.

Imperial Cancer Research Fund Jan 1985-Sept 1993
Biological Therapy Laboratory (Head Fran Balkwill)
44 Lincoln's Inn Fields
London WC2A 3PX

Scientific Officer/Senior Scientific Officer/Post Doctoral Scientist

I was exceptionally fortunate to have spent a considerable proportion of my early career at ICRF which has forged many links with research scientists, and clinical scientists. Many of these contacts continue and have helped develop research and clinical development programmes throughout my career. Many of these links are still active and my collaboration with Fran Balkwill still continues and I have been able to gain funding to help resource a post doctoral position in her new group.

During this phase of my career the research focus was very much in translational oncology with a specific focus on the role of cytokines in the biology of ovarian cancer. I was fortunate to continue to develop my academic career within this environment, initially undertaking a Masters degree in Immunology and then PhD. My PhD thesis focused on the role of TNF in the biology of ovarian cancer and led to a number of first author publications. My major contribution was to bring in molecular biology techniques to assess gene expression in primary tumour material. One key project success was the development of *in situ* hybridisation techniques for assessing cytokine mRNA expression. Development of this technology led to the creation of a new service department at the Central Laboratories ICRF.

Publications

Original papers

S. Naylor M J Smalley, D Robertson, B A. Gusterson, P A Edwards, T C Dale (2000) Retroviral expression of Wnt-1 and Wnt-7b produces different effects in mouse mammary epithelium J Cell Sci 113 2129-38

L Sarkar, M. Cobourne, **S. Naylor**, M Smalley, T Dale, P T Sharpe (2000) Wnt/Shh interactions regulate ectodermal boundary formation during mammalian tooth development. PNAS 97 4520-4

L.Griffiths, K. Binley, S. Iqball, O. Kan, P. Maxwell, P. Ratcliffe, C. Lewis, A. Harris, S. Kingsman, **S. Naylor** (2000). The macrophage-a novel system to deliver gene therapy to pathological hypoxia. Gene Therapy 7, 255-62

K. Binley, S. Iqball, A. Kingsman, S. Kingsman, **S. Naylor** (1999). An adenoviral vector regulated by hypoxia for the treatment of ischaemic disease Gene Therapy 10, 1721-27

K. Boast, K. Binley, S. Iqball, T. Price, H. Spearman, S. Kingsman, A. Kingsman, **S. Naylor** (1999) Characterisation of physiologically regulated vectors for the treatment of ischaemic disease. Human Gene Therapy 10, 2197-208

M.J. Smalley, F. Sara, H. Paterson, **S. Naylor**, D Cook, H. Jayatilake, L. G. Fryer, L. Hutchinson, M. J. Fry, T. C. Dale (1999) Interaction of axin and Dvl-2 proteins regulates Dvl-2-stimulated TCF-dependent transcription. EMBO J 18, 2823 - 2835

D. J. Phippard, S. J. Weber-Hall, P. T. Sharpe, **M .S. Naylor**, H. Jayatalake, R. Maas, I. Woo, D. Roberts-Clark, P. H. Frances-West, Y. Liu, R. Maxson, R. E. Hill and T.C.Dale (1996). Regulation of Msx-1, Msx-2, Bmp-2 and Bmp-4 during foetal and postnatal mammary gland development. Development 122, 2729-2737

D.W. Miles, L. Happerfield, **M.S. Naylor**, L.G. Bobrow, R.D. Rubens, F.R. Balkwill. (1994). Expression of TNF and its receptors in benign and malignant breast tissue. Int. J. Cancer. 56 777-782

M.S. Naylor, B. Davies, G.W. Stamp, F.R. Balkwill (1994). Expression and activity of matrix metalloproteinases and their inhibitors in ovarian cancer. Int. J. Cancer 58, 50-56

M.S. Naylor, G.W. Stamp, W.D. Foulkes, D.Eccles and F.R. Balkwill. (1993). Tumor necrosis factor and its receptors in human ovarian cancer-potential role in disease progression. J. Clin. Invest. 91 2194-2206

B. Davies, D.W. Miles, L. Happerfield, **M.S. Naylor**, L.G. Bobrow, R.D. Rubens, F.R. Balkwill. (1993). Activity of type IV collagenases in benign and malignant breast disease. Br. J. Cancer 67 1126-1131

M.S. Naylor, G.W. Stamp, W.D. Foulkes, D.Eccles and F.R. Balkwill (1993). Tumor necrosis factor and its receptors in human ovarian cancer-potential role in disease progression. J. immunol 150 292A

Michie CA, Scott KS, Happerfield L, **Naylor S**, Bobrow L, Beverley PC (1992). How significant is tumor-necrosis-factor-alpha secretion in acute-inflammation.
J Pathol 168 A 129

M.S. Naylor, G. W. H. Stamp, F. R. Balkwill (1990). Demonstration of mRNA for tumour necrosis factor and other cytokines in human colorectal cancer. Cancer Research 50 4436-4440

M.S. Naylor, S. T. A. Malik, G. W. H. Stamp, T. Jopling, F. R. Balkwill (1990) In situ detection of tumour necrosis factor in human ovarian cancer. [illegible]

S T A Malik, **M.S. Naylor** N East A Oliff F R Balkwill (1990) Cells secreting tumour necrosis factor show enhanced metastasis in nude mice Eur J Cancer 26 1031-1034

F R Balkwill, **M.S. Naylor** S T A Malik (1990) TNF as an anticancer agent Eur J Cancer 26 (5) 641-644

G B Dealtry **M.S. Naylor** W Fiers F R Balkwill (1987) The effect of recombinant human tumour necrosis factor on growth and macromolecular synthesis of human epithelial cells Exptl Cell Res 170 428-430

G B Dealtry, **M.S. Naylor**, W Fiers F R Balkwill (1987) DNA fragmentation and cytotoxicity caused by tumour necrosis factor is enhanced by interferon gamma Eur J Immunol 17 689-693

F R Balkwill, F Burke, **M.S. Naylor** D Talbot J Tavernier R Osborne H Durbin, W Fiers (1987) Evidence for tumour necrosis factor/cachectin production in cancer Lancet Nov 28th 1229

Book chapters/reviews

M.S. Naylor, Burke, F., and F. R. Balkwill (1995) Cytokines and Ovarian Cancer: Ovarian Cancer 3: ed F. Sharp, P. Mason, T. Blackett, J. Berek. Chapman Hall London

B Davies, **M.S. Naylor**, and F R Balkwill (1995) Matrix metalloproteases and their role in ovarian cancer: Ovarian Cancer 3, ed F Sharp P Mason, T Blackett, J Berek Chapman Hall London

M.S Naylor, M. Rolf, F. R. Balkwill (1995) Northern ribonuclease protection analysis and *in situ* hybridisation to cytokine mRNA In " Cytokines : A Practical Approach 2nd Ed" IRL Press Ed F R Balkwill.

F. Burke, **M.S. Naylor**, B.Davies and F R. Balkwill (1993) The Cytokine Wall Chart Immunology Today 14 165-170

S. Malik, **M.S. Naylor**, and F.R. Balkwill. (1992) Cytokines and Ovarian Cancer: Ovarian Cancer 2 : Biology, diagnosis and management, ed F. Sharp, W P Mason W Creasman.

S T A. Malik, D. B. Griffin, **M.S. Naylor**, W Fiers, A. Oliff, F.R. Balkwill (1990) The complex effects of recombinant TNF in human ovarian cancer xenograft models. In 'Cytokines and Lipocortins in Inflammation and Differentiation'. Wiley-Liss Inc. Ed. Melli and Parente 393-403.

M.S. Naylor and F. R. Balkwill.(1990) Northern Blotting and *In Situ* Hybridisation To Cytokine mRNA. In " Cytokines : A Practical Approach " IRL Press Ed. F. R. Balkwill.

Membership of Societies

British Association of Cancer Research
American Association of Cancer Research

I have acted as referee for the following journals

American Journal of Obstetrics and Gynecology
British Journal of Cancer
Developmental Biology
European Journal of Cancer
Experimental Cell Research
The Histochemical Journal
Journal of Pathology
Gene Therapy

Education

1974-1981	Royal Grammar School High Wycombe. Bucks.	10 O-levels 3 A-levels
1981-1984	University of Warwick	B.Sc Hons Microbiology and Virology Class 2(ii)
1986-1988	KQC, University of London	M.Sc. Immunology
1988-1992	ICRF Central Laboratories	PhD Thesis TNF and Ovarian Cancer Supervisor F.R. Balkwill